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NOTE

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Karyotype analysis of interspecific fusants of basidiomycetes by pulsed-field gel electrophoresis

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Abstract The purpose of this research was to analyze the karyotype of the interspecific fusants of two *Pleurotus* species. Auxotrophic mutants derived from the cultivated strain of *P. ostreatus* and *P. cornucopiae* were used. Protoplasts were fused electrically, and the fusants were selected under auxotrophic complementation. Esterase isozyme analysis showed that several fusants had isozyme bands originating from both parental strains, and others had unilateral isozyme bands. The fusant that had expressed isozyme bands of both parental strains showed chromosomal DNA bands of both of the parental strains in pulsed-field gel electrophoresis analysis. Despite the above results, the chromosomal composition of the fusants obtained by the pulsed-field gel electrophoresis did not exhibit all of the bands of both fusion parents.

Key words *Pleurotus* species · Protoplast fusion · Electrophoretic karyotype · Chromosome · Mushroom

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Introduction

Among the fungi, edible mushrooms have been favored as well as cultivated by Japanese people for more than a hundred years. Like other crops, the mushrooms have also been genetically improved. Recently, the cell fusion technique has been applied to edible mushrooms.

Several authors[1–5] have reported cell fusion of the fungi using polyethyleneglycol (PEG) as a fusogenic agent. Using PEG, some authors[6–9] performed intraspecific protoplast fusion and others[8,10–12] interspecific protoplast fusion of edible mushrooms. However, the method using a chemical fusogen has drawbacks, such as low fusion frequency, which is due to the cytotoxicity of the fusogenic agent, or difficulty in controlling the many parameters effecting the fusion.

Electrofusion was first introduced by Senda et al.[13] Zimmermann[14] improved it so it was more suitable for practical use. The method employs electric fields of a high-frequency alternating current (AC) and a short direct-current (DC) pulse. These electrical parameters can be controlled through visual inspection of the fusion process under the microscope. This is a major advantage of electrofusion over the fusion method using PEG. Another advantage is that the method employs no cytotoxic agent for fusion treatment. Some authors[15–21] have applied the electrofusion process to the genetic improvement of fungi. The reports are on the yeast *Aspergillus*, the zygomycete *Abisidia*, and the edible mushrooms *Pleurotus* and *Auricularia*.

The purpose of our research was to analyze the karyotype of the interspecific fusants derived by electrofusion of the two edible mushrooms *Pleurotus ostreatus* and *Pleurotus cornucopiae*.

Materials and methods

Organisms

Auxotrophic mutants of *Pleurotus ostreatus* (strain Po4-7-203) and *Pleurotus cornucopiae* (strain F450-8-162) were

used in this research. These mutants had been induced by Yokono et al.[22] from monokaryotic strains using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). The required nutrients for the mutants were thymine (or uracil) and adenine, respectively. The strains were maintained on potato-dextrose-agar (PDA).

Isolation of protoplasts

Each strain was grown on SMY agar medium (1% sucrose, 1% malt extract, 0.4% yeast extract, and 1.5% agar) for 2 weeks. A portion of mycelium was cut and inoculated into the SMY liquid medium. The culture was stirred once a day with a magnetic stirrer for 10 min. After 10 days' incubation at 23°C, the mycelium was digested in an enzyme solution (2% Cellulase Onozuka R-10 and 1% Novozyme 234) for 2 h at 30°C. The resultant protoplasts were separated from the mycelial fragments and washed 3 times with a fusion medium (0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.5 M mannitol). For protoplast fusion, protoplasts from each strain were mixed at a ratio of 1:1. The suspension density of the protoplasts was adjusted to 1.2×10^7/ml to 1.2×10^8/ml.

Protoplast fusion

Protoplast fusion was performed by the application of an AC field that was interrupted by DC pulses. The intensity of the AC field was 200–250 V/cm. The intensity of DC pulses was 1–3 kV/cm. The duration of the DC pulses was 40 μs. Five to thirty DC pulses were applied to achieve protoplast fusion. Then the AC field was reduced to 100 V/cm and applied continuously for 5 min before being cut off. The protoplasts subjected to fusion treatment were recovered and plated onto minimal medium (MM: 0.15% ammonium phosphate dibasic, 2% glucose, 0.046% potassium phosphate monobasic, 0.1% potassium phosphate dibasic, 0.05% magnesium sulfate anhydrous, 1.5% agar, 0.5 M sucrose, and 0.000012% thiamine hydrochloride). After several weeks of incubation, the regenerated colonies on the MM were isolated and plated separately onto SMY agar plates. The regenerated colonies were presumed to be fusants.

Isozyme analysis

The recovered fusants were subjected to isozyme analysis for characterization. Polyacrylamide gel was used for the electrophoresis. The mycelia to be analyzed were grown for 14 days on SMY agar plates, after which 200 mg of each mycelium was harvested. The mycelia were washed with distilled water and homogenized by an ultrasonic homogenizer in extraction buffer (0.94% sodium phosphate monobasic dihydrate, 1.61% sodium phosphate dibasic heptahydrate, 0.015% dithiothreitol, and 19.81% sodium L-ascorbate). The homogenates were then centrifuged at 11750 *g* for 30 min. Supernatants were collected and used as a sample for the electrophoresis. Electrophoresis was performed in constant-current mode at 17 mA/gel. Detection of esterase enzyme was performed using α-naphthyl acetate as the substrate, as described by Kadomatsu.[23]

Pulsed-field gel electrophoresis

Contour-clamped homogeneous electric field (CHEF) electrophoresis was used for the experiment. Protoplasts of the fusants to be analyzed were prepared as described above and embedded in 0.5% low-melting-point agarose. The agarose block containing protoplasts was incubated in a solution consisting of 0.5 M EDTA, 50 mM Tris-NaOH pH 9.5, and 1% *N*-lauroyl sarcosinate for 5 min at room temperature. The block was then incubated in the same solution containing 0.2% proteinase K at 50°C for 24 h. Electrophoresis was performed as described by Tamai et al.[24] Briefly, 0.6% (w/v) Megarose and 0.5 × TBE run buffer were used with pulses of 360 s–100 V for 12 h, 660 s–75 V for 48 h, 1800 s–75 V for 12 h, 3600 s–50 V for 24 h. CHEF-DRII from Bio-Rad was used. The gel was stained with ethidium bromide 1 μg/ml for 1 h and destained by distilled water for another 1 h.

Results

Plating efficiency was estimated by plating protoplasts of the specific mutants onto complete medium (CM: SMY agar plus 0.5 M sucrose), onto nutrient-supplemented minimal medium (NMM: for Po4-7-203, MM plus thymine 20 μg/ml; for F450-8-162, MM plus adenine 20 μg/ml), and onto MM (Table 1). Protoplasts of 1.65×10^5 were plated onto each plate.

The frequency of back-mutation was estimated by dividing the plating efficiency on MM by that on NMM. Neither strain formed a colony on MM. The analysis was repeated with a higher concentration of protoplasts, and frequencies of back-mutation for these strains were estimated to be less than 8.3×10^{-10} for Po4-7-203 and less than 1.0×10^{-8} for F480-8-162. The plating efficiency of the protoplasts after the fusion treatment was 0.000012%. Fusion frequency was 0.00075%. Fusion frequency was estimated by:

(Plating efficiency on MM after fusion treatment)/
(average of plating efficiency of each mutant on CM)

Among the regenerants after fusion treatment, colonies that were well separated from others were transferred onto

Table 1. Plating efficiency of the mutant strain

Strain	Plating efficiency (%)		
	CM	NMM	MM
P4-7-203	0.07	0.26	0.0
F450-8-162	3.12	0.14	0.0

CM, complete medium; NMM, nutrient-supplemented minimal medium; MM, minimal medium

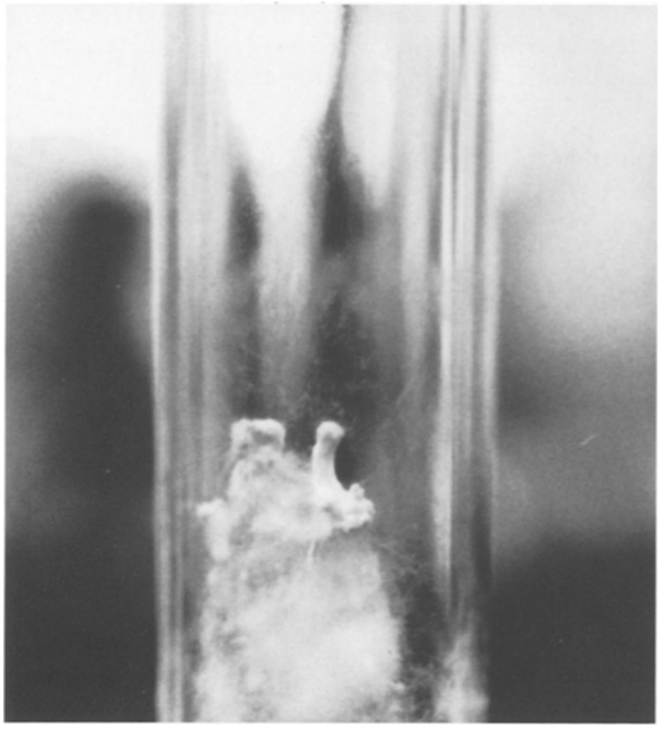

Fig. 1. Primordium formed on a fusant between *Pleurotus cornucopiae* F450-8-162 and *Pleurotus ostreatus* Po4-7-203

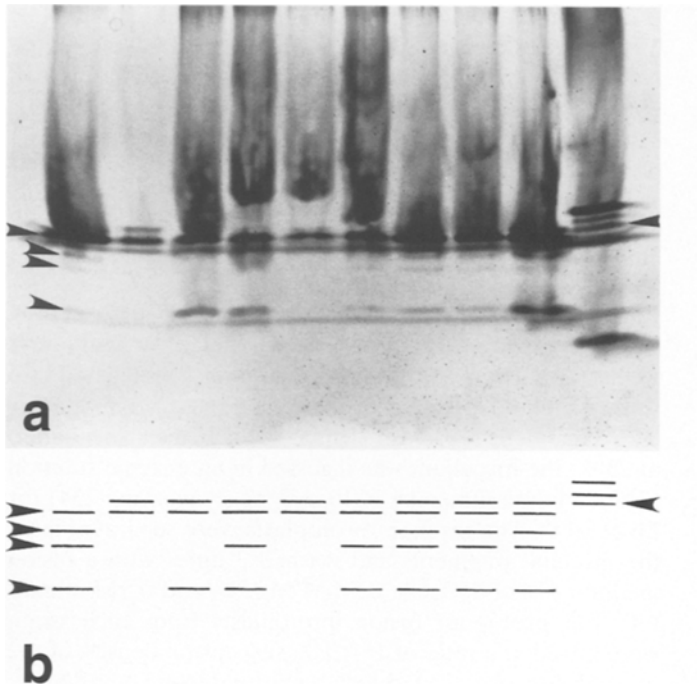

Fig. 2. Esterase zymograms of *Pleurotus cornucopiae* F450-8-162 (fusants Fu77, Fu78, Fu145, Fu150, Fu151, Fu173, Fu177, Fu196) and *Pleurotus ostreatus* Po4-7-203 (from left side). *Arrowheads* indicate bands that also appeared in fusants

a fresh SMY medium for further analysis. From the fusion between Po4-7-203 and F450-8-162 a total of 76 colonies were isolated. Microscopic observations showed no clamp connection in any of the 76 isolates. Twenty-four isolates formed primordia while they were being maintained on the SMY agar slants (Fig. 1).

Esterase isozyme analysis was performed on the isolates. Some isolates showed bands that also appear in both *P. ostreatus* and *P. cornucopiae*. These isolates showed one band that appear in Po4-7-203 and one or a few bands that appear in F450-8-162 (Fig. 2). This fact indicates that the isolates were fusants between Po4-7-203 and F450-8-162. There were also isolates that showed bands of only one of the fusion parents (data not shown).

Seven isolates from the fusants that showed isozyme bands of both parents were subjected to CHEF electrophoresis (Fig. 3). All the isolates had all of the DNA bands of F450-8-162. In addition to the DNA bands of F450-8-162, one isolate (Fu77) had a DNA band of Po4-7-203.

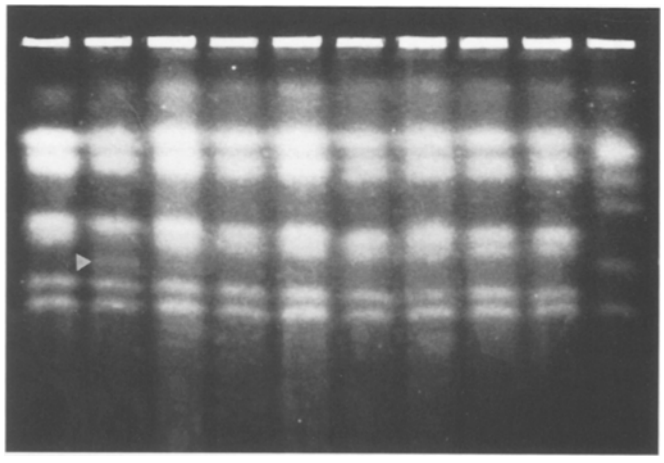

Fig. 3. Pulsed-field electrophoresis pattern of *Pleurotus cornucopiae* F450-8-162 (fusants Fu77, F78, Fu145, Fu150, Fu151, Fu173, Fu177, Fu196) and *Pleurotus ostreatus* Po4-7-203 (from left side). *Arrowhead* indicates the band of Po-4-7-203 that appeared in Fu77

Discussion

In their communications on intraspecific fusions, most authors[2-5] have reported higher fusion frequencies than that derived from the current experiment. Hashiba and Yamada[2] reported a fusion frequency of 1%–2% for interspecific fusion of *Rhizoctonia solani*. Kiguchi and Yanagi[3] reported a fusion frequency of 1%–4% for intraspecific fusion of *Coprinus machrorhizus*. Ohba et al.[4] reported a fusion frequency of 0.5%–3.4% for common-B mating-type intraspecific fusion of *Coprinus cinereus*. Toyomasu et al.[5] reported a fusion frequency of 1.5% for intraspecific fusion of *Coprinus cinereus*. The fusion frequency derived in current experiment was 0.00075%. The reason for the difference of the fusion frequency can be attributed to the difference between intraspecific fusion and interspecific

fusion. The fusion frequency of interspecific fusion is presumed to be lower than that of intraspecific fusion.

Clamp connection was not observed in any of the isolates. Tamai et al.[20] reported that they found pseudo clump-like structures but no clamp connection. Although the possibility of mutation in the gene involved in the formation of the clamp connection cannot be excluded, the results of the current experiment may indicate that formation of the clamp connection occurs only under a specific combination of the species of parental strains.

The same isolates showed bands that also appeared in both *P. ostreatus* and *P. cornucopiae* for esterase isozyme analysis. Toyomasu et al.,[5] reported that bands of both of the fusion parents were seen in the fusant of *Coprinus cinereus*. Ohmasa[7] reported similar results for *Pleurotus ostreatus*. Toyomasu and Mori,[12] and Tamai et al.[20] reported similar results for *Pleurotus* species, and Sunagawa[21] reported similar result for *Auricularia* species. In the present experiment, there were also isolates that showed bands of only one of the fusion parents. Toyomasu and Mori[12] reported similar results. Because auxotrophic complementations were shown clearly on the regeneration plates, these isolates were considered to be recombinant monokaryons of the corresponding fusion parent strains.

Primordia that formed on the isolates of the fusant indicate the activation of the genes that take part in the formation of fruiting bodies. The probability that the activation occurred in the monokaryotization of the dikaryotic hyphae through protoplast formation can be excluded because neither the mutants nor the monokaryotic hyphae have exhibited primordia for several vegetative generations, even though they were cultured in the same manner as the present isolates.

Several authors[25,26] have used pulsed-field gel electrophoresis (PFGE) for karyotype analysis of the microorganism. The method allows separation of large DNA molecules on agarose gel by means of changing the direction of the electric field during the run. One of the isolates (Fu77) showed bands of both fusion parents in PFGE. The isolate (Fu77) showed isozyme bands of both fusion parents with esterase isozyme analysis. However, the isolate (Fu77), which is several vegetative generations removed from the fusion plate, could not grow on MM. Because the isolate (Fu77) showed only one band of *P. ostreatus* in PFGE, the phenomenon of its not growing on MM indicates that the isolate (Fu77) is not a heterokaryon but a monokaryon that happened to contain chromosomes of another parental strain. The phenomenon may also indicate that the chromosomes of the *P. ostreatus* Po4-7-203 that contains a gene needed for the auxotrophic complementation was excluded from the isolate. This is due to the fact that, if auxotrophic complementation had been achieved by recombination of the gene itself, it is unlikely to revert in a short period of time.

Alternatively, some isolates showed PFGE patterns identical to that of F450-8-162 and could grow on MM (Fu78, Fu150, Fu151, Fu173). This phenomenon indicates that these isolates were recombinants.

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